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Crystal structure of the sweet-tasting protein thaumatin II at 1.27 Å

Running title: Structure of thaumatin II at 1.27 Å

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charge**

Abstract

Thaumatococcus, an intensely sweet-tasting protein, elicits a sweet taste sensation at 50 nM. Here the X-ray crystallographic structure of one of its variants, thaumatococcus II, was determined at a resolution of 1.27 Å. Overall structure of thaumatococcus II is similar to thaumatococcus I, but a slight shift of the Cα atom of G96 in thaumatococcus II was observed. Furthermore, the side chain of residue 67 in thaumatococcus II is highly disordered. Since residue 67 is one of two residues critical to the sweetness of thaumatococcus, the present results suggested that the critical positive charges at positions 67 and 82 are disordered and the flexibility and fluctuation of these side chains would be suitable for interaction of thaumatococcus molecules with sweet receptors.

2 1. Introduction

Thaumatococcus daniellii Benth, a plant native to tropical West Africa [1].

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structural basis of the sweetness of thaumatin is not fully understood. In spite of the feasibility of producing crystals of thaumatin, studies have focused on the structure of thaumatin I, the most abundant component of plant thaumatin. While various crystal forms of thaumatin I (monoclinic, orthorhombic, tetragonal, and hexagonal) have been examined extensively [4-7], little attention has been paid to the structure of thaumatin II. Since thaumatin has great potential as a low-calorie sugar substitute, it is worth obtaining structural information on thaumatin II as well as thaumatin I. Variations at position 67, critical for sweetness (Lys67 for thaumatin I and Arg67 for thaumatin II), are particularly interesting. Herein the structure of thaumatin II was determined at a resolution of 1.27 Å.

2. Materials and methods

2.1. Purification of thaumatin II

Powdered thaumatin (Wako Pure Chemical Industries Ltd., Osaka, Japan) was dissolved in 5 mM sodium phosphate buffer, pH 7.0, the supernatant obtained by centrifugation (8,000 ×g) was applied to an SP-Sephadex C-25 cation exchange column, previously equilibrated with 5

1 mM sodium phosphate buffer, pH 7.0, and the bound proteins were eluted
2 with a linear gradient of 20 to 120 mM NaCl in 5 mM sodium phosphate
3 buffer, pH 7.0. Fractions containing thaumatin II, detected by SDS-PAGE
4 and native PAGE, were combined and precipitated with 75% ammonium
5 sulfate. The precipitate was collected by centrifugation at 8,000×g,
6 dissolved in 20 mM Hepes buffer, pH 7.0, containing 150 mM NaCl, and
7 further purified by gel-filtration chromatography (HW50F, Tosoh Co.,
8 Tokyo, Japan). The purified thaumatin II was concentrated by a Centricon
9 10 (Millipore, Bedford, MA), and the protein concentration was measured
10 with a NanoDrop®ND-1000 spectrophotometer (NanoDrop Technologies,
11 Inc., Rockland, DE).

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13 2.2. Crystallization and X-ray diffraction

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15 Crystallization was performed by using the hanging-drop
16 vapor-diffusion method. The hanging drops were prepared with 5 µL of
17 30-50 mg/mL protein solutions and 5 µL of a reservoir solution consisting
18 of 0.1M N-(2-Acetanido) iminodiacetic acid, 0.5-1.0 M potassium sodium
19 tartrate, pH 6.5, and 25% glycerol. Data were collected at 100K using an
20 ADSC quantum CCD detector with synchrotron radiation at a wavelength

of 1.0 Å at the BL-44XU station of SPring-8 (Hyogo, Japan). Diffraction data obtained at up to 1.27 Å (Table 1) were processed, merged, and scaled from 50 to 1.27 Å using the HKL2000 program package [8].

2.3. Structure determination and refinement

The structure of thaumatin II was determined by molecular replacement using the program Shelxpro [9] with the previously reported thaumatin structure as a reference (PDB entry 3al7) [7]. The rigid body refinement and subsequent refinement were performed using the SHELXL program with the data for up to 1.5 Å. $|F_o| - |F_c|$ and $2|F_o| - |F_c|$ maps were used to obtain the correct model. Water molecules were incorporated where the difference in density exhibited values of more than 3.0σ above the mean and the $2|F_o| - |F_c|$ map showed a density of more than 1.0σ .

Model-building was performed using the Coot program [10]. The residues Asn46, Ser63, Lys67, and Arg76 were substituted with Lys, Arg, Arg, and Gln, respectively. The occupancy of the major conformation was refined first, then the second conformation was assigned and refined based on its $|F_o| - |F_c|$ map, and finally atoms of all conformations were refined. The occupancies of the disorders were treated as free variables and refined

using the FVAR restraints. Refinement of the model with isotropic B -factor against the data up to a resolution of 1.27 Å by SHELXL resulted in an R_{work} of 16.76% and an R_{free} of 19.29%. Subsequent anisotropic B -factor refinement against the data up to 1.27 Å lowered the R_{work} and R_{free} to 11.09% and 14.62%, respectively. To finalize the refinement, the model was refined against all data for 40 cycles using conjugate-gradient least squares minimization, leading to an $R1$ of 11.13% for 67,833 reflections and 10.55% for 64,442 reflections with $F_o > 4\sigma(F_o)$ in the resolution range 10–1.27 Å (Table 1). The quality of the structure was assessed by using PROCHECK [11] and WHAT IF [12]. The electron density maps and structural images were generated using PyMOL [13]. The coordinates and observed intensities of plant thaumatin II have been deposited in the PDBj (accession code 3aok).

3. Results and discussion

3.1. Properties of thaumatin II

Plant thaumatin comprises a mixture of at least five variants designated thaumatin I, thaumatin II, thaumatin a, thaumatin b, and

thaumatin c [1]. Ion exchange column chromatography and native-PAGE revealed about 60% of the plant sample to be thaumatin I and 20% is thaumatin II. The sequencing of thaumatin I and thaumatin II showed that four positions (N46K, S63R, K67R, and R76Q) varied [14, 15] (Fig. 1A). In the presence of sodium potassium tartrate ions, thaumatin easily crystallizes as a tetragonal system. Although only four residues differed between thaumatin I and thaumatin II, the crystals of thaumatin II measured approximately $0.1 \times 0.1 \times 0.3$ mm, much smaller than those of thaumatin I ($0.3 \times 0.3 \times 1.0$ mm) [16]. Since these four residues are located at the surface (Fig. 1A), the net charge might have affected the nucleation of the crystals.

3.2. Overall structure of thaumatin II

The final model of thaumatin II consists of 207 residues with a total of 2,215 protein atoms, 2 tartrate ions, 4 glycerol molecules, and 414 water molecules. A single thaumatin molecule is contained in an asymmetric unit. The R factor for the final structure is 10.55% for 64,442 reflections with $F_o > 4\sigma(F_o)$ and 11.13% for all data (67,833 reflections). A Ramachandran plot calculated for the final model showed that 89.3% of the amino acid

1 residues are in most favored regions, 10.1% are in additional allowed
 2 regions, and 0.6% are in generously allowed regions. No residues are in
 3 disallowed regions (Table 1). The only residue located in a generously
 4 allowed region was Asp25, part of the thaumatin loop
 5 (D21-A22-A23-L24-D25-A26), which was not observed in other
 6 non-sweet thaumatin-like proteins. The average *B* factor for α -carbon, side
 7 chain, and all protein atoms is 11.466 Å², 15.595 Å², and 17.957 Å²
 8 respectively. The Matthews coefficient *V_m* [17], and the solvent content are
 9 calculated to be 2.81 Å³/Da and 56.2%, respectively (Table 1). The four
 10 amino acid residues (Lys46, Arg63, Arg67, and Gln76) which differed from
 11 those of thaumatin I are all located on the molecular surface (Fig. 1A). The
 12 electron density maps around these residues are shown in Fig. 2. Most of
 13 the side chains are well fitted compared to those of thaumatin I. Of note,
 14 however, Arg67, which is critical for sweetness, is disordered and has two
 15 alternative side chains (Fig. 1B and Fig. 2C). Although it has been
 16 suggested that the basicity of the side chain of residue 67 is responsible for
 17 elicitation of the sweet taste, the results of the present study further
 18 confirmed that the distribution of the positively charged patches formed by
 19 guanidino-groups is not rigid but flexible (Fig. 1B). Since the threshold
 20 value of sweetness for thaumatin II is similar to that for thaumatin I [18],

the flexibility and fluctuation of the positively charged side chain at residue 67 would be suitable for interaction with the sweet receptors.

3.3. Comparison of *thaumatin II* with *thaumatin I*

Superposing the coordinates of the C α atoms of *thaumatin II* onto the recombinant *thaumatin I* (PDB entry 3a17) more clearly illustrates the structural differences among the two variants (Fig. 3A). Amino acid residues with C α carbons having root-mean square deviations (r.m.s.d.) that were above 0.2 Å were Y57, G96, R119, G141, G142, G143, T161, G162, and K163 (Fig. 3B). Among them, Y57, R119 and K163 were modeled in two alternative conformations and highly disordered. Furthermore, G162, K163 and R119 are considered to be influenced by solvents such as glycerol [5]. Although the three consecutive glycine residues from positions 142 to 144 are highly disordered in any *thaumatin* structure, the r.m.s.d. value of the G96 of *thaumatin II* was relatively high and this difference might be specific to *thaumatin I* and *II*. As to G96, the r.m.s.d. values were also investigated against the structures of *thaumatin I* (PDB entries 1rqw, 2vhk, 1lxz, and 3ald) which had been determined before at high resolutions (ranging from 0.94 to 1.25 Å). The results showed

that r.m.s.d. values against thaumatin II were 0.28 Å, 0.35 Å, 0.39 Å, and 0.26 Å, respectively, suggesting that the difference of C α atom in G96 might be specific to thaumatin II. Since the side chain of K46 in thaumatin II, N46 in thaumatin I, is adjacent to Q94 and Y95 (4.0 to 6.5 Å away from K46), the difference in the properties of the side-chain (N and K) might influence the crystal packing and result in a slight shift of the C α atom of G96.

The structural requirements for the sweetness of thaumatin molecules have been investigated extensively [2, 3, 19-21]. Although the three-dimensional structure of sweet receptors has not yet been elucidated, models of the binding between sweet-tasting proteins and sweet receptors have been reported [22-30]. Understanding the structural determinants of thaumatin's sweetness will provide valuable insights into the mechanisms by which sweet taste is perceived.

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3 2009A1096, 2009B1379, 2010A6538, 2010B1064).

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Legends to Figures

Figure 1. (A) Overall structure (stereo diagram) of thaumatin II. The four residues (Lys46, Arg63, Arg67 and Gln76) which differ from thaumatin I are shown in a stick model (blue). The residue critical for sweetness (Arg82) is also shown in a stick model (green). (B and C) Comparison of thaumatin II with thaumatin I at two critical residues (67 and 82). Side chains are represented by a stick model with grey (thaumatin I) and red (thaumatin II). (B) residue 67 and (C) residue 82.

Figure 2. The model and electron density maps around Lys46, Arg63, Arg67 and Gln76 in thaumatin II. (A) Lys46. (B) Arg63. (C) Arg67. (D) Gln76. The σ_A -weighted $2mFo-DFc$ maps contoured at 1.0σ are shown in blue and $mFo-DFc$ maps contoured at 3.0σ and -3.0σ are shown in green and red, respectively.

Figure 3. The superposition of the coordinates of the $C\alpha$ atoms of thaumatin II onto the recombinant thaumatin I (PDB entry 3al7). (A) Histograms of r.m.s.d. values of $C\alpha$ atoms with the residue number. (B)

1 The residues with r.m.s.d. values greater than 0.2 Å (Y57, G96, R119,
2 G141, G142, G143, T161, G162, and K163) are shown in red in a ribbon
3 model of thaumatin II.

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5 **Legends to tables**

6

7 **Table 1. Data collection and refinement statistics**

8 Data on the highest shells are given in parentheses.

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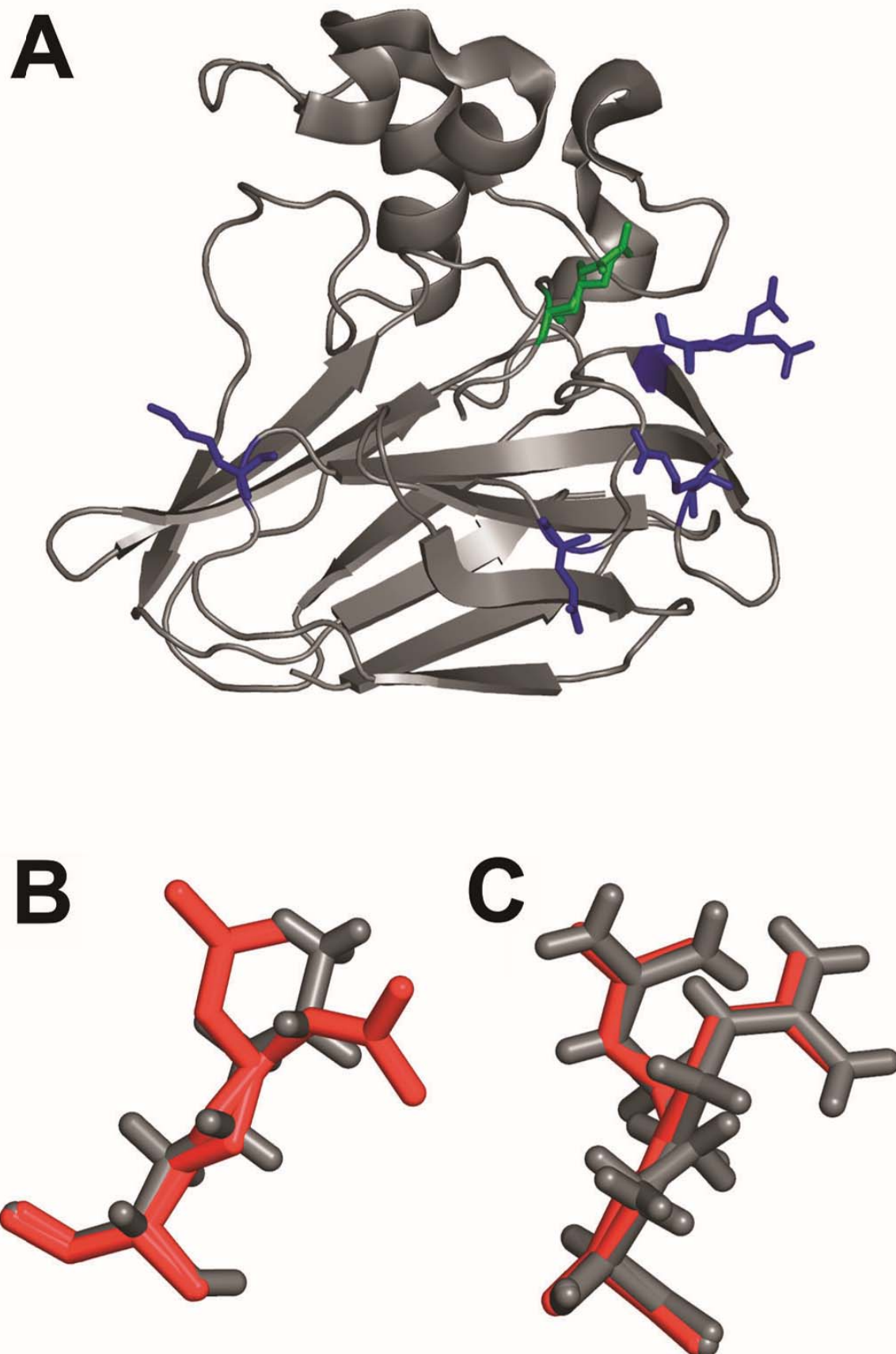


Figure 1.

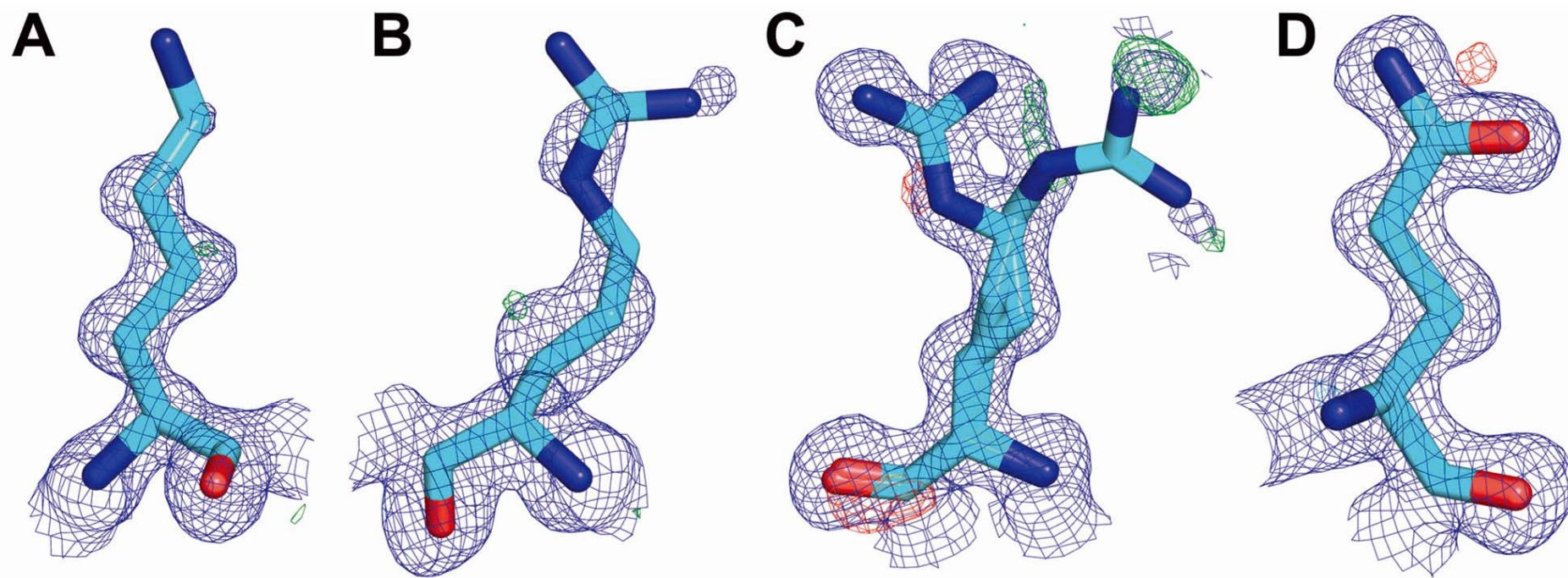


Figure 2.

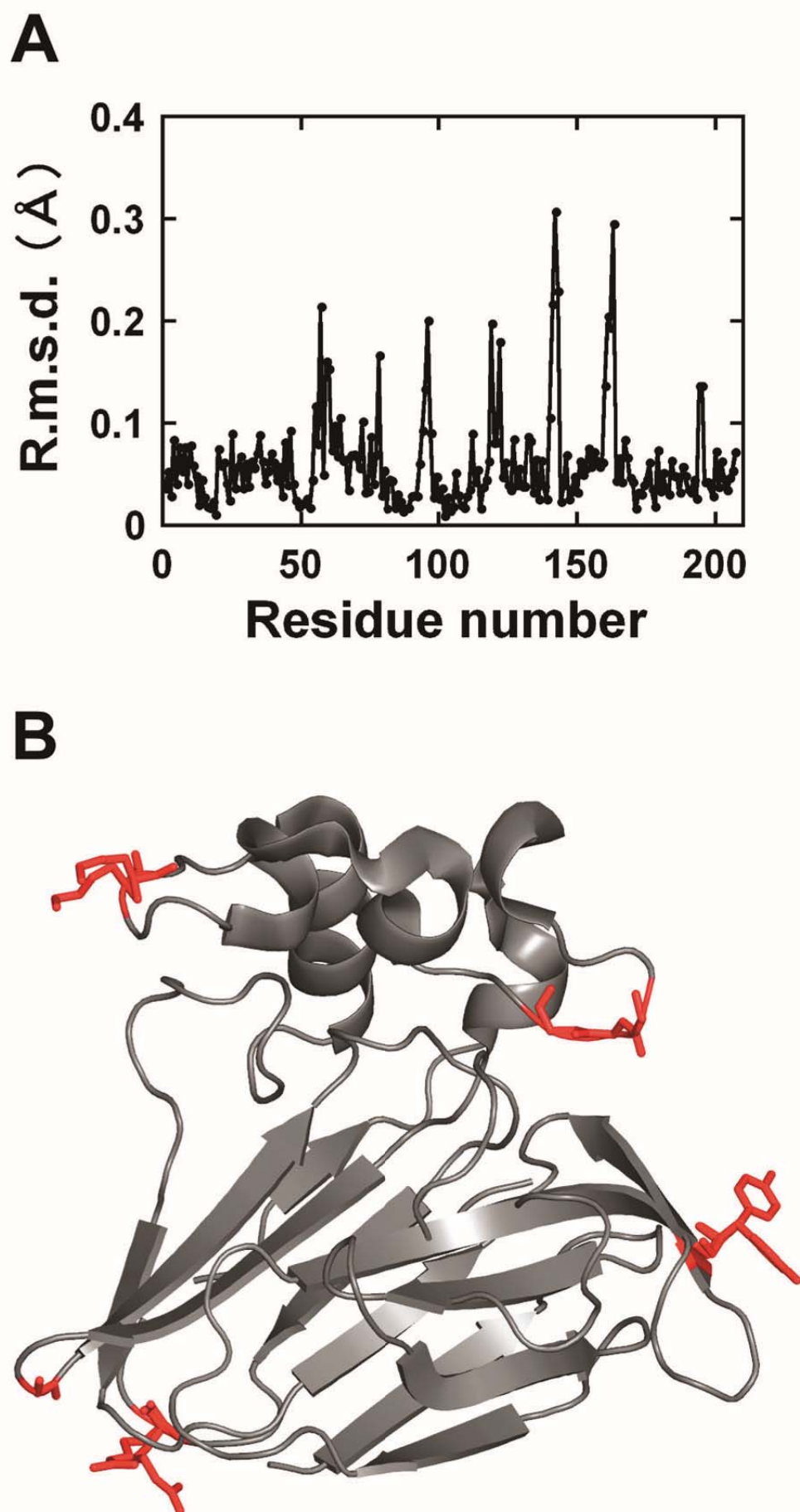


Figure 3.

Table 1. Diffraction data and refinement statistics

Thaumatococcus	
<i>Data collection</i>	
Beamline	SPring-8 BL44XU
Detector	ADSC Quantum 210
Crystal system	Tetragonal
Space group	$P4_12_12$
Cell dimensions (Å, °)	a = 57.769, b = 57.769, c = 150.126, $\alpha = \beta = \gamma = 90$
X-ray wavelength (Å)	1.0
Temperature (K)	100
Resolution limit (Å)	50.0-1.27 (1.32-1.27)
Total reflections	942,463
Unique reflections	68,167
R_{merge}	0.072 (0.328)
Completeness (%)	100 (100)
Redundancy	9.1 (8.8)
$\langle I \rangle / \langle \sigma(I) \rangle$	48.1 (8.3)
<i>Refinement</i>	
Resolution (Å)	10-1.27
Unique reflections	67,833 ($F_o > 4\sigma$: 64,442)
Isotropic: $R_{\text{work}}/R_{\text{free}}$ (%)	16.76/19.29 (15.99/18.43)
Anisotropic: $R_{\text{work}}/R_{\text{free}}$ (%)	11.09/14.62 (10.51/13.85)
R_{cryst} (%)	11.13 (10.55)
Average B factor	17.96
Protein main chain	11.47
Protein side chain	15.60
Tartrate	17.57
Glycerol	32.30
Water	35.74
R.m.s.deviation from ideal values	
r.m.s.d bond (Å)	0.015
r.m.s.d angle (Å)	0.033
Ramachandran plot (%)	
Most favored	89.3
Additional allowed	10.1
Generously allowed	0.6
Disallowed	0
Matthews coefficient Vm (Å ³ /Da)	2.81
Solvent content (%)	56.2